

CpA dinucleotide, the adenine of said dinucleotide hybridizing to a uracil which, before bisulfite treatment, had been a cytosine that comprised a CpG dinucleotide.

The diagram below shows implementation of these rules to select a primer that can be used to amplify clone 2.B.53 of the NotI/EcoRV library (Table I and attached sequence listing).

- 5 In the diagram, I shows the end of the 2.B.53 clone containing the methylation-sensitive NotI site (NotI recognition sequence is shown in bold letters). CpG dinucleotides are shaded. To amplify a region of this clone rightward of the NotI site, the first primer is identical to the top strand of the duplex shown in I. However, since bisulfite treatment of the DNA in I converts cytosines to uracils, guanines within the PCR primer must be replaced with adenines. II shows the sequence
- 10 of the bottom strand of I after bisulfite treatment converts cytosines to uracils. A primer complementary to the bisulfite-treated bottom strand has the sequence shown in III.

I

	CG	CGCG		CG	CG
	GC	GCGC		GC	GC

5' **GCGCGCGCG** GTTAGCTTCTCCTGTC **AA** CAGGG-----

15 3' **CGCGCGCG** CAATCGAAGAGGACAG **TT** GTCCC-----

II

3' UGUUGGUGUUAATUGAAGAGGAUAGGUTTGUGTUUU-----

20 III

5' ACAACCACAATTAACCTTCTCCTATCCAAACA 3'

III shows the entire sequence of one of the two primers used to amplify unmethylated genome DNA corresponding to library clone 2.B.53. This primer encompasses 5 CpG dinucleotides, as shown by the shading in I above. Encompassment of 2 or more such CpG dinucleotides is preferred so that this primer will not hybridize to a bisulfite-treated template which contains methylated cytosines. The 3' end of the primer shown in III ends in a CpA dinucleotide. This is also preferred in order to provide maximal discrimination of the primer between methylated and unmethylated template DNA in MSR. The primer shown in III has a

30 length of 31 nucleotides.

The second primer is designed to work with the first primer in PCR amplification such that a fragment of less than about 200 base pairs is amplified. Therefore, this primer is made to a sequence rightward of the sequence shown in I. The sequence of this primer is complementary in sequence to the strand of the template which has its 5' end as part of the methylation-sensitive

restriction enzyme site, except that guanine residues are replaced with adenine residues. This primer is preferably between 15 and 32 nucleotides in length. This primer is also designed to preferably encompass 2 or more CpG dinucleotides. Preferably, the 3' end of said primer ends with a CpA dinucleotide.

5 The diagram below shows implementation of these rules to select a primer that can be used to amplify unmethylated genome DNA corresponding to clone 2.B.53 of the NotI/EcoRV library. IV shows a region of the 2.B.53 clone about 70 nucleotides rightward of the sequence in I of the earlier diagram. The CpG dinucleotides within the sequence are shaded. To amplify a region leftward of this region, this second primer must be complementary to the top strand of the

10 duplex shown in IV. However, bisulfite treatment of the DNA in IV converts cytosines to uracils. A primer complementary to this bisulfite-treated top strand has the sequence shown in VI.



VI shows the entire sequence of the second primer used to amplify unmethylated genome DNA corresponding to library clone 2.B.53. This primer encompasses 8 CpG dinucleotides, as shown by the shading in IV. Encompassment of 2 or more such CpG dinucleotides is preferred. The 3' end of the primer shown in VI ends in a CpA dinucleotide. This is also preferred. The primer shown in VI has a length of 31 nucleotides. Together, the first and second primers amplify a PCR fragment of 128 base pairs in length.

30 The above primers amplify genome DNA that does not contain 5-methylcytosine. The above primers will not amplify genome DNA containing 5-methylcytosines because 5-methylcytosines are not converted to uracils by bisulfite treatment. The two primers already described (III and VI), therefore, will not be complementary to bisulfite-treated genome DNA which is methylated.

Therefore, a second set of primers is designed to amplify genome DNA that is methylated. Methylation in human cells occurs at cytosines that are part of CpG residues. Such methylated cytosines are not converted to uracil by bisulfite treatment. Cytosines that are not part of CpG residues are not methylated and, therefore, are converted to uracil by bisulfite. The

- 5 primers of the second set are designed to amplify the same region of a library clone as did the first set of primers. But, because the genome DNA contains both cytosines that are methylated and cytosines that are not methylated, the sequences of primers used to amplify such DNA are different than the sequences of the first primer set. Like the first set of primers, however, the primers of the second set are preferably between 15 and 32 nucleotides in length. Preferably the

- 10 3' ends of such primers contain CpG dinucleotides.

The diagram below shows implementation of these rules to select the first of two primers that can be used to amplify methylated genomic DNA corresponding to clone 2.B.53 of the NotI/EcoRV library. In the diagram below, VII shows the end of the 2.B.53 clone containing the NotI site (NotI recognition sequence is bolded). CpG dinucleotides are shaded. Cytosines within said CpG dinucleotides are methylated and are underlined in VII to indicate methylation to 5-methylcytosine. Treatment of the DNA in VII with bisulfite produces a bottom strand with the sequence shown in VIII. In VIII, only unmethylated cytosines are converted to uracil by bisulfite.

20 VII

CG	CGCG		CG	CG
<u>GC</u>	<u>GC</u> GC		GC	<u>GC</u>

5' GGCGCGC GTTAGCTTCTCCTGTC AA CAGGG-----

3' CCGCGC CAATCGAAGAGGACAG TT GTCCC-----

VIII

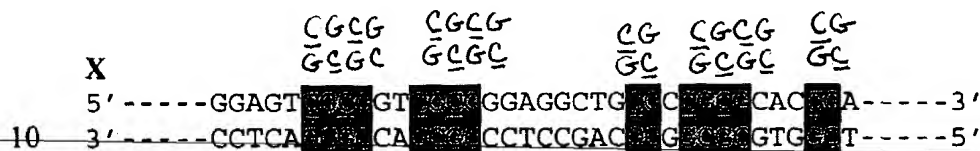
25 3' UGCUGGCGCUAATUGAAGAGGAUAGGCTTGC GTUUU-----

IX

5' ACGACCGCGATTAACTTCTCCTATCCGAACG 3'

- 30 A primer complementary to the bisulfite-treated bottom strand shown in VIII is shown in IX. Said primer will prime PCR amplification of sequences rightward of those shown in VII. The primer shown in IX encompasses 5 CpG dinucleotides. Encompassment of 2 or more such CpG dinucleotides is preferred. The 3' end of the primer shown in IX ends in CpG. This is also preferred. The primer shown in IX has a length of 31 nucleotides.

A second primer is designed to work with the primer shown in IX to amplify methylated genome template DNA. Design of such a primer is shown below. In the diagram, X shows the same region of clone 2.B.53 (approximately 70 nucleotides rightward of the sequences shown in VII) that is shown in IV. Treatment of the DNA in X with bisulfite produces a top strand with the sequence shown in XI. In XI, only unmethylated cytosines are converted to uracil by bisulfite.



A primer complementary to the bisulfite-treated top strand (XI) has the sequence shown in XII. Said primer will prime PCR amplification of sequences leftward of those shown in X. The primer shown in XII encompasses 8 CpG dinucleotides. Encompassment of 2 or more such CpG dinucleotides is preferred. The 3' end of the primer shown in XII ends in a CpG dinucleotide. This is also preferred. The primer shown in XII has a length of 31 nucleotides. Together, the first (IX) and second primers (XII) of the second set amplify a PCR fragment of 128 base pairs in length.

Example 4. Use of oligonucleotides to diagnose cancer

The library clones, and DNA sequences within, can be used to detect DNA methylation in a genome at the specific sequences identified by the sequences within the clone. Such detection can be diagnostic for cancer. Various methods can be used for such diagnosis.

A. Diagnosis of cancer using methylation-sensitive restriction enzymes followed by Southern blot

Cleavage or lack of cleavage by a methylation-sensitive restriction enzyme at a specific restriction enzyme recognition site can be detected by a probe for the specific recognition site,